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Original communication

Determination of ethyl glucuronide in human hair by hydrophilic interaction liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

Ethyl glucuronide (EtG) is a direct metabolite of ethanol and has been utilized as a marker for alcohol intake. This study presents development, validation and application of a new hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS) method for the analysis of EtG in human hair samples. The linearity was assessed in the range of 5–2000 pg/mg hair, with a correlation coefficient of >0.99. The method was selective and sensitive, with a limit of detection (LOD) and limit of quantitation (LOQ) of 0.05 pg/mg and 0.18 pg/mg in hair, respectively. Differently from the extraction procedures in the literature, a fast and simple liquid–liquid method was used and highest recoveries and cleanest extracts were obtained.

The method was successfully applied to 30 human hair samples which were taken from those who state they consume alcohol. EtG concentrations in the hair samples of alcohol users participated in this study, ranged between 1.34 and 82.73 pg/mg. From the concentration of EtG in hair strands 20 of the 30 subjects can be considered regular moderate drinkers.

1. Introduction

Overconsumption of alcohol is a contributing factor medico-legal case.[1] It is a fact that alcohol is a contributing factor for traffic accidents, serious traumas, natural deaths and the other medico-legal cases. Furthermore, alcoholism is one of the most frequent addictions. In such cases, the most frequently used samples for ethanol analysis are blood, urine, saliva, and breath. Additionally, compared to urine or blood analysis, hair analysis offers a much larger window of detection for alcohol consumption. Ethyl glucuronide (EtG) is a product of the non-oxidative ethanol metabolism. EtG is a nonvolatile, water-soluble, direct metabolite of ethanol, which can be detected in body fluids, tissues and hair. Hair analysis has become an important diagnostic tool for the determination of chronic alcohol consumption since EtG can accumulate in hair. Its first identification was reported by Sachs et al., in 1993.[2] Several methods to determine EtG concentration in human hair have been recently developed.[3–7]

Previously conducted studies have utilized analytical methods based on liquid chromatography (LC) with mass spectrometry (MS).[8] LC–MS/MS methods do not need derivatizations, and are thus less complex and time consuming. Recently, the use of hydrophilic interaction liquid chromatography (HILIC) coupled to MS, developed for the separation of polar compound. HILIC was applied successfully for the analysis of EtG in hair.[9,10]

The aims of this study were to determine EtG in human hair samples using HILIC, and to find out the correlation between alcohol intake and EtG levels in hair samples.

2. Materials and methods

2.1. Hair samples

Thirty hair samples included in the study belonged to males aged between 25 and 63 years with a mean age of 41.77 and median age of 42.50 years. A written informed consent to participate in the study was obtained from all the individuals. Before collecting the

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Table 1
Matrix effect.

Analyte	Theoretical concentration (ng/ml)	Matrix effect (%)	Relative matrix effect RSD (%)
EtG	20	105	3.5
	500	100	2.7

hair samples, each participant was requested to complete an anonymous questionnaire. This included a series of questions such as daily intake of ethanol, age, and sex. The study was approved by the Ethics Committee of Cukurova University. Hair specimens were collected by fixing a strand of hair on the posterior region of vertex and by cutting it as close to the skin as possible. Blank hair was obtained from young children free from alcohol. The samples were stored in aluminum foil until analyses.

2.2. Chemicals and reagents

EtG and d_5-EtG were purchased from Medichem (Germany). Methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade or highest purity available.

Stock solutions of EtG (1 mg/ml) and d_5-EtG (0.5 mg/ml) were prepared in methanol and they were stored at −20 °C. Working standard solutions used for calibration and quality control samples were prepared by 0.015, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 5 and 10 μg/ml. All working solutions were stored in a refrigerator when not in use.

2.3. Sample preparation

Hair samples were washed twice using water (5 ml). Each strand was cut into small pieces (>1 mm) by scissors and 200 mg of the hair was weighted in 10-ml conical-bottom glass tubes and spiked with 50 μl internal standard solution (d_5-EtG, 2.5 mg/l in methanol). The samples were vortexed for 1 min and samples were ultrasonicated for 15 min in 2 ml acetonitrile/water solution (80:20), centrifuged (3500 rpm for 5 min). Next, the supernatant was filtrated by nylon filter and injected into the LC–MS/MS.

2.4. Instrumentation

Chromatographic separation was carried out with an LC system comprised of an Agilent 1200 series binary pump, vacuum degasser and auto-sampler (Agilent, Germany). The analyte and internal standard were eluted on a Zorbax Hilic Plus, Rapid Resolution (50 mm × 4.6 mm, 3.5 μm, Agilent, USA). Mobile phase composed by (A) ammonium acetate 1 mM in water and (B) acetonitrile. The separation was achieved by isocratic elution. The injection volume 10 μl was used in all cases.

Analyses were performed on an Agilent (USA) triple quadruple mass spectrometer model 6460, equipped with an atmospheric pressure pneumatically assisted electrospray ionization source. The interface was operated in negative ionization mode. High purity nitrogen served both as collision-induced dissociation gas, drying gas (set at 11 L/min) and nebulizer gas (set at 50 psi). The source temperature was maintained at 350 °C, the capillary voltage was set at 4000 V and the fragment or voltage at 135 V after optimization by injecting EtG standard. Analysis was performed by multi-reaction monitoring, using 221 → 75, 221 → 85 amu for EtG and 226 → 75, 226 → 85 amu for d_5-EtG.

2.5. Validation

The method was validated for selectivity, linearity and sensitivity, precision and accuracy and for recovery.

Seven-point calibration curves were prepared by spiking the weighted amount of blank hair with standard solution to obtain the concentration range of 0–5–20–50–100–500–1000–2000 pg/mg for EtG. To assess linearity, a calibration curve was prepared in replicate ($n = 3$) and analyzed. The acceptance criteria were: a correlation coefficient (r) and determination coefficient (r^2) > 0.99, and a precision and accuracy, for the back calculated concentrations of the calibration points, within ±15%.

The limit of detection (LOD) was 0.05 pg/mg, with an S/N ratio of 3. The limit of quantitation (LOQ) was 0.18 pg/mg. Under the chromatographic conditions used, there was no interference with the analytes by chemicals or any extractable endogenous materials present in hair.

The extraction recovery tests were performed by analyzing extracted hair samples and diluted standard solutions at equal concentrations. Five replications at low and high concentrations were prepared. The recoveries at 20 and 1000 pg/mg were 97.84% and 100%, respectively.

The matrix effect was evaluated according to the following procedure[11]: the analyte signal in the spiked water was compared with the analyte signal in the matrix fortified after extraction and the matrix effect percentage was calculated as % = (extracted matrix height/water height) × 100. Five replicates of hair extracts were analyzed (Table 1).

3. Results and discussion

The chromatograms of blank hair sample and of a blank hair sample spiked with EtG (20 pg/mg) concentration, extracted as described above and separated in HILIC conditions, are presented in Fig. 1.

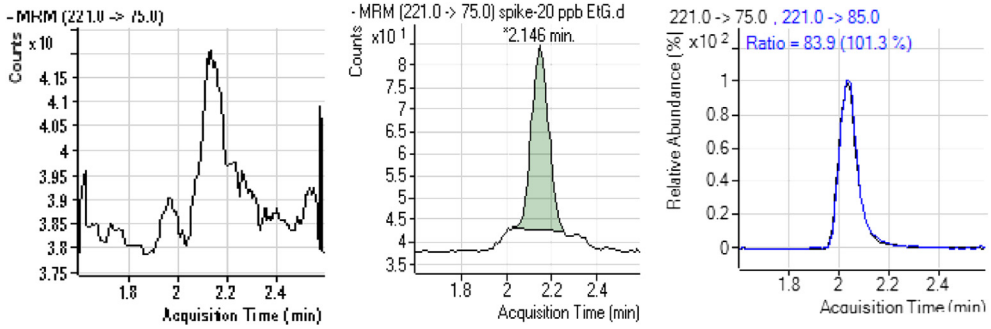

Fig. 1. Chromatograms of blank hair sample and a blank spiked with EtG at 20 pg/mg.

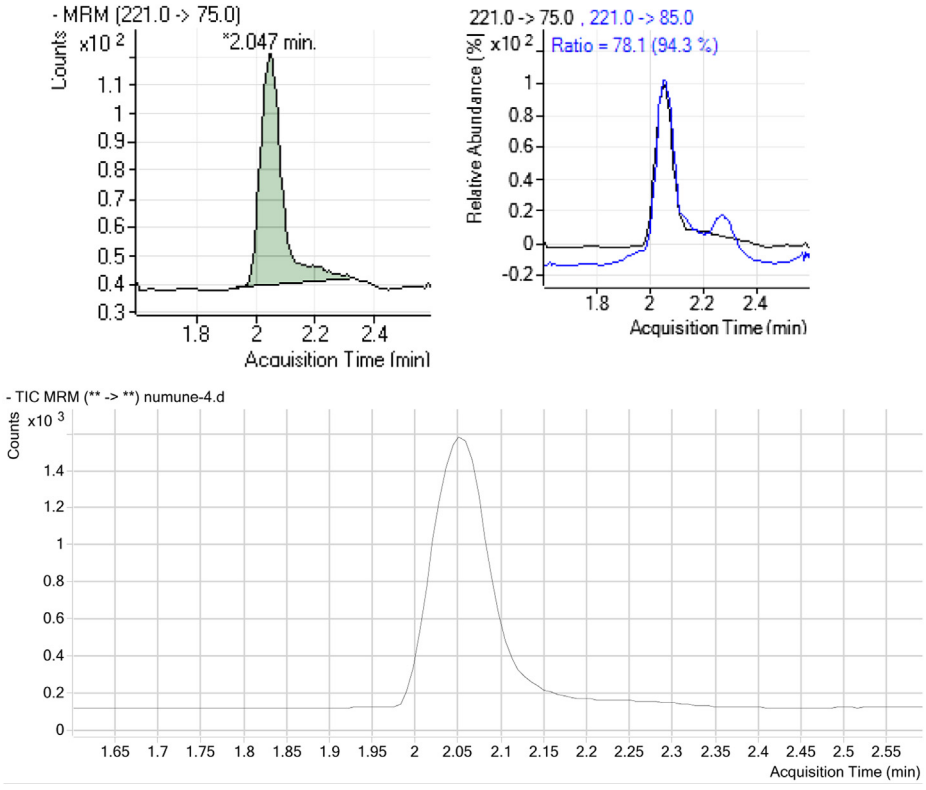

Fig. 2. Chromatogram of a real sample (EtG: 82.73 pg/mg).

A total of 30 human hair samples were analyzed for EtG. The EtG concentrations were between 1.34 and 82.73 pg/mg (mean 13.5 ± 15.25 pg/mg, median 9.5 ± 15.25 pg/mg). Fig. 2 shows the chromatogram obtained after extraction of a hair from an alcohol user.

EtG, a metabolite of ethanol, is a valuable marker for alcohol intake; thus, it is of high importance in forensic and clinical cases involving alcohol. Skipper et al.[12] point out that a prerequisite for a reliable test is the ability to show the use of alcohol at least in a few days after its consumption. According to a report by WHO, men who take at least 40 g of alcohol per day and women who use at least 20 g of alcohol are accepted as heavy drinkers.[13]

The aim of the hair test is to reveal the suspicious alcohol use and determine the consumption of alcohol by the people who are previously known to be alcohol users. The difference between social drinkers and alcohol addicts is specified on the basis of EtG cut-off values. In order to avoid different interpretations done by different laboratories, the Society of Hair Analysis (2012) reported the value of EtG cut-off as 7 pg/mg.[14] The concentrations above this value show repeated alcohol consumption. In our study, only twelve samples showed concentrations above 7 pg/mg. The other values were found to be below the cut-off value. This clearly shows that those people consumed alcohol repeatedly.

Pragst et al.[15] detected EtG in hair samples obtained from three heavy drinkers. Similarly, Alt et al.[16] identified EtG concentrations ranging between 218 and 4025 pg/mg, in 16 postmortem hair samples of alcohol addicts. They also found that EtG concentrations ranged between 119 and 388 pg/mg in the patients under treatment. However, they found no evidence of EtG in the hair samples of children and of social drinkers who consume alcohol below 20 g per day.

The mean value of EtG concentrations in the currently studied hair samples was 13.5 ± 15.25 pg/mg. The common characteristics of these samples were that these people were social drinkers. The differences in the concentrations of EtG in hair samples was related to daily consumption of alcohol as shown by Spearman correlation coefficient ($r = 0.668$, $p < 0.001$) as shown in Fig. 3. These findings were compatible with those of by Politi et al.,[13] Appenzeller et al.[7] and Kronstrand et al.[17]; however, no correlation between the amount of consumed alcohol and the EtG concentrations was observed in hair samples, in the study by Janda et al.[18] One of our study limitations was participant's hair color because some studies[7,19] have also shown that hair pigmentation does not influence incorporation of EtG in hair therefore hair color was not included in this study.

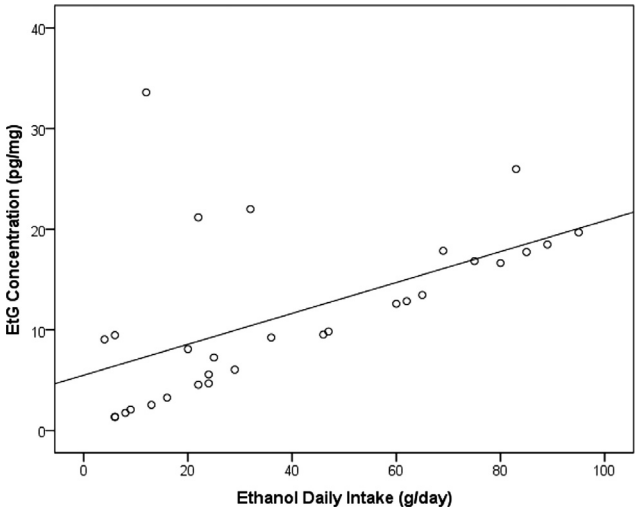

Fig. 3. Relationship between EtG concentration and ethanol daily intake.

In the present study, we demonstrated the successful determination of EtG levels by LC/MS/MS using a HILIC column. However, for EtG, which is an extremely polar compound, the use of hydrophilic column was thought to be preferable. Through HILIC column use, the detection of a very low level of EtG could be achieved (LOQ: 0.18 pg/mg) in hair samples.

4. Conclusion

In this study, a selective and sensitive identification and quantification method for EtG in human hair samples was developed using HILIC/MS/MS. The method was applied to real samples and was able to rapidly quantify EtG also in the hair samples of alcohol users.

Ethical approval
None.

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Conflict of interest
None.

Competing interests
There are no competing interests for any of authors.

Contributorship
All authors included in the authors list have contributed to the data collection, data evaluation, writing of manuscript, language use etc. And no professionals other than authors contributed to the any process of during article preparation.

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